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PATENT COOPERATION TREATY 9/914880

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

LOCK, Graham
Fry Heath & Spence
The Old College
53 High Street
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ROYAUME-UNI

Date of mailing (day/month/year)
05 September 2001 (05.09.01)

Applicant's or agent's file reference
NO 6316/WO

IMPORTANT NOTIFICATION

International application No.
PCT/EP00/01743

International filing date (day/month/year)
01 March 2000 (01.03.00)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

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2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

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3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
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Authorized officer
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PATENT COOPERATION TR .TY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 20 October 2000 (20.10.00)	
International application No. PCT/EP00/01743	**Applicant's or agent's file reference** NO 6316/WO
International filing date (day/month/year) 01 March 2000 (01.03.00)	**Priority date** (day/month/year) 01 March 1999 (01.03.99)
Applicant SHER, Alexander et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

19 September 2000 (19.09.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Manu Berrod
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09/914880

PATENT COOPERATION TREATY

REC'D 25 MAY 2001
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PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70) 14

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
NO 6316/WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/01743	01/03/2000	01/03/1999
International Patent Classification (IPC) or national classification and IPC A23L1/304		
Applicant SOCIETE DES PRODUITS NESTLE S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
19/09/2000	22.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Rinaldi, F Telephone No. +49 89 2399 7360

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/01743

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1,2,4-14	as originally filed	
3	with telefax of	08/05/2001

Claims, No.:

1-13	with telefax of	08/05/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:
   - ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-13
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-12
	No:	Claims	13
Industrial applicability (IA)	Yes:	Claims	1-13
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

R Item I

Basis of the report

The amendments fulfill the requirements of Art.34(2)(b) PCT.

Re Item V

Reasoned statement under Art.35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Novelty

1 The presently claimed subject-matter is not anticipated by the prior art (see below) (Art.33(2) PCT).

Inventive step

2 The subject-matter of independent product claims 1, 8, 9, and 11 and of the thereof depending claims 2-7, 10 and 12 fulfills the requirements of Art.33(3) PCT.

2.1 **D2 (=WO 98 48648)** discloses iron fortification systems in foodstuff or beverages comprising iron(II) compounds which are chelated by amino-acids or oligopeptides consisting of 2-4 amino-acids (p.6 l.19-p.7 l.16). The oligopeptide combinations are not limited as to the sequence of amino acids. Said fortification systems provide good bioavailability while preventing off-flavour and discolouring in the food products in which they are used (p.3 l.7-27).

2.2 Complexes as disclosed in present claims 1-12 differ in that the polypeptide

- has a molecular weight between 2000-6000 and
- that it is obtainable from partially hydrolysed egg white protein.

2.3 The objective technical problem is to provide an alternative iron fortification system and a process for its manufacture.

2.4 The prior art teaches away from using peptides consisting of more than 4 amino acids. In fact, single amino acids as ligands are most preferred (D2, claim 22). The man skilled in the art had no indication, even if he is only looking for an alternative solution to the technical problem, to provide an iron fortification complex wherein ferrous iron is chelated by hydrolysed egg white protein having a molecular weight of 2000-6000, i.e. wherein the polypeptide chain consists roughly of 2060 amino acids.

3 The subject-matter of independent process claim 13 does not fulfill the requirements of Art.33(3) PCT.

3.1 Iron fortification systems comprising iron(II) which are chelated with amino-acids or oligopeptides consisting of 2 to 4 amino acids are known from D2 (see above).

3.2 The presently claimed process differs in that dipeptides, tripeptides, tetrapeptides, oligopeptides or polypeptides are obtained from egg white protein.

3.3 The objective technical problem is to provide a process for the manufacture of iron-protein fortification systems as above, wherein the protein can be, according to the wording of the claim, any one of dipeptide, tripeptide, tetrapeptide, oligopeptide or polypeptide.

3.4 No inventive step can be seen in producing di-, tri- or tetrapeptides as obtainable by enzymatically digesting egg white protein. The formation of the complex is trivial in view of the disclosures in the example of **D3 (=WO 93/08830)**.

Re Item VIII

Certain observations on the international application

1 For reasons of clarity the attention of the applicant is drawn to the following (Art.6 PCT).

1.1 The present set of claims is not fully supported by the description (p.3 l.34-p.4 l.21) (Art.6 PCT).

1.2 The expression "about" used throughout the description and especially throughout the claims is regarded to be vague (PCT IPE Guidelines Section IV III-4.5a).

1.3 Present claims 7 is redundant in view of claim 5.

but heat coagulated form. The complex is recovered as a precipitate. However, when these iron albumin complexes are used in beverages, discolouration and oxidation does occur. For example, chocolate beverages fortified with iron albumin complexes turn a gray color.

More recent examples of iron complexes are described in US patent 3,969,540 where iron in the ferric form is complexed with hydrolysed casein or hydrolysed liver powder. Various other hydrolysed proteins are also mentioned as possible ligands. The complexes are collected as insoluble precipitates. Unfortunately the iron in the complexes is unlikely to have acceptable bioavilability.

Another example of an iron complex is described in WO 98/48648. This document discloses iron (II) compounds chelated with amino acids or oligopeptides of two to four amino acids.

Further examples iron complexes are described in US patent 4,172,072 where iron is complexed with substantially completely hydrolysed collagen. Various other completely hydrolysed proteins are also mentioned as possible ligands. However, the complexes are stated to be stable under acidic conditions and, since the conditions in the gut are acidic, the iron in the complexes is unlikely to have acceptable bioavailability. Also, the complexes are not sufficiently strong to prevent discolouration and lipid oxidation.

Further examples are described in US patent 4,216,144 where iron in the ferrous form is complexed with hydrolysed protein; especially soy protein. The bioavailability of the iron in the complexes is claimed to be better than ferrous sulfate. However, when ferrous-soy hydrolysates complexes are used in beverages, discolouration and oxidation does occur. For example, chocolate beverages fortified with ferrous-soy hydrolysates complexes turn a gray color.

Other examples of iron complexes are described in Japanese patent applications 2-083333 and 2-083400. In these applications, ferrous caseinate complexes are used to treat anemia. However, these complexes are not suitable for use in fortifying foods and beverages because they are not sufficiently stable. Also, these complexes are in the form of coagulates and are difficult to disperse.

It is therefore an object of the invention to provide an iron fortification system which is relatively stable but in which the iron is relatively bioavailable.

Summary of the Invention

Accordingly, in one aspect, this invention provides an iron-protein hydrolysate complex which comprises ferrous ions chelated to partially

CLAIMS

1. An iron-protein hydrolysate complex which comprises ferrous ions chelated to partially hydrolyzed egg white protein having a molecular weight in the range of about 2'000 to about 6'000.

2. A complex according to claim 1 in which the partially hydrolyzed egg white protein is microbial protease hydrolysate.

3. A complex according to claim 2 in which the microbial protease is obtained from *Aspergillus oryzae* and contains both endo-peptidase and exo-peptidase.

4. A complex according to claim 1 in which partially hydrolyzed egg white protein is a microbial protease hydrolysate obtained by hydrolyzing egg white protein with a protease obtained from *Aspergillus oryzae* and containing both endo-peptidase and exo-peptidase, and a protease obtained from *Bacillus licheniformis* and containing endo-proteinase.

5. A complex according to claim 1 which contains about 1% to about 2% or about 4.5% to about 10% by dried weight of ferrous ions.

6. A complex according to claim 1 which is stable at neutral pH but disassociates at a pH below about 3.

7. A complex according to any preceding claim which comprises about 1% to about 2% or about 4.5% to about 10% by dried weight of ferrous ions.

8. A sterilized liquid beverage which contains lipid and a stable iron fortification system, the iron fortification system comprising an iron-protein hydrolysate complex according to any preceding claim.

9. A sterilized liquid beverage which contains polyphenols and a stable iron fortification system, the iron fortification system comprising an iron-protein hydrolysate complex according to any one of claims 1 to 7.

10. A beverage according to claim 9 which is a tea beverage.

11. A beverage powder which contains lipid and a stable iron fortification system, the iron fortification system comprising an iron-protein hydrolysate complex according to any one of claims 1 to 7.

12. A beverage powder according to claim 11 which contains cocoa.

13. A process for preparing an iron fortification system, including an iron-protein hydrolysate complex according to any one of claims 1 to 7, the process comprising:

enzymatically hydrolyzing an egg white protein using a microbial protease to provide a partially hydrolyzed egg white protein:

adding a ferrous source to the partially hydrolyzed egg white protein under acidic conditions; and

raising the pH to 6.5 to 7.5 for forming a ferrous-hydrolyzed egg white protein complex as the iron fortification system.

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Iron Fortification System

Field of the Invention

This invention relates to an iron fortification system which is based upon hydrolysates of egg white protein and which may be used in foods and beverages. The invention also relates to a method of preparing the system and to fortifying foods and beverages with iron.

Background of the Invention

Iron is an essential trace element in animal and human nutrition. It is a component of heme in hemoglobin and of myoglobin, cytochromes and several enzymes. The main role of iron is its participation in the transport, storage and utilization of oxygen. Inadequate iron is a direct cause of the high incidence of anemia, especially among children, adolescents and women. The need for adequate iron is one which extends for the entire life of the human being.

However the body does not produce iron and is totally dependent on an external supply of iron; nutritional or supplementary. The recommended daily allowance for iron intake is usually about 10 mg per day. However the amount needed is dependent on age and sex. Children, women up to the time of menopause, and expectant and nursing mothers have higher requirements of iron.

Therefore iron deficiency is essentially a nutritional problem; a nutritional problem which is common not only in the developing countries. The problem is readily dealt with by consuming foods which naturally provide adequate iron but this is not always possible in disadvantaged societies. Also, many foods normally consumed in developed countries are poor in iron.

To provide a source of iron, many foods and beverages are supplemented with iron. Usually the iron source used in supplementation is a soluble iron salt such as ferrous sulfate, ferrous lactate, ferrous gluconate, ferrous fumarate, ferric citrate, ferric choline citrate, and ferric ammonim citrate. Ferrous sulfate is especially common due to its good bioavailability. Unfortunately, iron supplementation and especially ferrous sulfate supplementation has deleterious effects. In particular, the iron often causes discoloration and off-flavors due to its capacity to interact with polyphenols and lipids and to promote destructive free-

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radical reactions. This is especially the case at high temperatures and in the presence of oxygen and light.

For example, the addition of a soluble iron source to chocolate milk powder causes the beverage to turn to dark gray when reconstituted with water or milk. It is believed that this is due to the interaction between the iron and iron sensitive ingredients, such as polyphenols. Further, the addition of soluble iron sources to milk, cereals, other fat containing products, mostly products with high level of unsaturated fatty acids, causes flavor changes due to lipid oxidation. Lipid oxidation not only affects the organoleptic properties of foods and beverages, but also undesirably affects the nutritional quality of these products. These interactions can be also enhanced during heat treatment, such as pasteurization or sterilization. In addition, the pH of some iron salts systems may not be compatible with other ingredients or may affect the flavor. Also, from a technical point of view, soluble iron salts can cause corrosion of processing equipment.

Unfortunately, non-soluble or slightly soluble iron sources such as elemental iron, ferric pyrophosphate, etc., are not sufficiently bioavailable. Therefore, while they may cause little or no discoloration and off-flavor problems, they are poorly absorbed by the body.

To deal with these problems, there have been several attempts to encapsulate or complex soluble iron sources in a way which reduces their reactivity but which maintains their bioavailability. However the attempts have not been entirely successful.

An example of encapsulated iron source is described in US patent 3,992,555 where iron is coated in an edible, metabolizable fat which has a melting point between about 38°C and about 121°C. Hydrogenated and refined vegetable oils, and particularly distilled monoglycerides from fully hydrogenated cottonseed oil, are described to be suitable. Although this encapsulation of the iron results in about a 20% reduction in bioavailability, this is stated to be acceptable providing the iron source used has a sufficiently good bioavailability. However, the primary problem is that, if the foods must undergo any form of harsh processing, the capsule is destroyed. Consequently the encapsulated iron cannot be used in products which need to be retorted or subjected to other forms of harsh treatment.

An early example of an iron complex is described in US patent No 505,986. This complex is an iron albumin preparation. The albumin is in intact

but heat coagulated form. The complex is recovered as a precipitate. However, when these iron albumin complexes are used in beverages, discoloration and oxidation does occur. For example, chocolate beverages fortified with iron albumin complexes turn a gray color.

More recent examples of iron complexes are described in US patent 3,969,540 where iron in the ferric form is complexed with hydrolyzed casein or hydrolyzed liver powder. Various other hydrolyzed proteins are also mentioned as possible ligands. The complexes are collected as insoluble precipitates. Unfortunately the iron in the complexes is unlikely to have acceptable bioavailability.

Further examples iron complexes are described in US patent 4,172,072 where iron is complexed with substantially completely hydrolyzed collagen. Various other completely hydrolyzed proteins are also mentioned as possible ligands. However, the complexes are stated to be stable under acidic conditions and, since the conditions in the gut are acidic, the iron in the complexes is unlikely to have acceptable bioavailability. Also, the complexes are not sufficiently strong to prevent discoloration and lipid oxidation.

Further examples are described in US patent 4,216,144 where iron in the ferrous form is complexed with hydrolyzed protein; especially soy protein. The bioavailability of the iron in the complexes is claimed to be better than ferrous sulfate. However, when ferrous-soy hydrolysate complexes are used in beverages, discoloration and oxidation does occur. For example, chocolate beverages fortified with ferrous-soy hydrolysate complexes turn a gray color.

Other examples of iron complexes are described in Japanese patent applications 2-083333 and 2-083400. In these applications, ferrous caseinate complexes are used to treat anemia. However, these complexes are not suitable for use in fortifying foods and beverages because they are not sufficiently stable. Also, these complexes are in the form of coagulates and are difficult to disperse.

It is therefore an object of the invention to provide an iron fortification system which is relatively stable but in which the iron is relatively bioavailable.

Summary of the Invention

Accordingly, in one aspect, this invention provides an iron-protein hydrolysate complex which comprises ferrous ions chelated to partially

hydrolyzed egg white protein having a molecular weight in the range of about 500 to about 10'000.

It is surprisingly discovered that complexes formed from iron in the ferrous form and partially hydrolyzed egg white protein are very stable. In fact, the complexes are sufficiently stable as to be suitable for use in retorted products which contain lipids and polyphenols. However, despite the stability, the iron in the complexes has substantially the same bioavailability as ferrous sulfate; which is remarkably good.

Preferably, the partially hydrolyzed egg white protein has a molecular weight in the range of about 2'000 to about 6'000.

In another aspect, this invention provides an iron-protein hydrolysate complex which comprises ferrous ions chelated to egg white protein which is partially hydrolyzed using a microbial protease.

Preferably, the microbial protease is a fungal protease obtained from *Aspergillus oryzae* and contains both endo-peptidase and exo-peptidase.

In a further aspect, this invention provides an iron-protein hydrolysate complex which comprises ferrous ions chelated to partially hydrolyzed egg white protein; the complex containing about 1% to about 2% or about 4.5% to about 10% by dried weight of ferrous ions.

The complexes are preferably stable at neutral pH but disassociate at a pH below about 3.

In a yet further aspect, this invention provides a sterilized liquid beverage which contains lipid and a stable iron fortification system, the iron fortification system comprising an iron-protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein. The beverage may be a chocolate containing beverage.

In yet another aspect, this invention provides a sterilized liquid beverage which contains polyphenols and a stable iron fortification system, the iron fortification system comprising an iron-protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein. The beverage may be a tea beverage.

The beverages may be sterilized by retorting or ulta high temperature pasteurization.

The invention also provides a beverage powder which contains lipid and a stable iron fortification system, the iron fortification system comprising an iron-

protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein. The beverage powder may contains chocolate.

In a further aspect, this invention provides a process for preparing an iron fortification system, the process comprising:

enzymatically hydrolyzing, preferably under acidic conditions, an egg white protein using a microbial, preferably an acidic fungal, protease to provide a partially hydrolyzed egg white protein;

adding a ferrous source to the partially hydrolyzed egg white protein under acidic conditions; and

raising the pH to 6.5 to 7.5 for forming a ferrous-hydrolyzed egg white protein complex as the iron fortification system.

The partially hydrolyzed egg white protein may be subjected to further hydrolysis steps prior to the addition of the ferrous source. Preferably the fungal protease is obtained from *Aspergillus oryzae* and contains both endo-peptidase and exo-peptidase.

The process may also include the further step of drying the ferrous-hydrolyzed egg white protein complex to a powder form.

Detailed Description of the Preferred Embodiments

Embodiments of the invention are now described by way of example only.

This invention is based upon the discovery that partially hydrolyzed egg white protein is able to strongly complex with ferrous ions and yet provide the iron in a bioavailable form. The resulting iron complexes have reduced ability to cause deleterious effects such as lipid oxidation, color degradation, and vitamin C degradation. This makes the iron complexes an ideal vehicle for fortifying foods and beverages; especially foods and beverages intended to improve nutritional status.

The iron source that may be used in the iron complexes may be any food grade ferrous salt, such as ferrous sulfate, ferrous chloride, ferrous nitrate, ferrous citrate, ferrous lactate, or ferrous fumarate, or mixtures thereof. However, the preferred iron source is ferrous sulfate. The iron source is preferably provided in the form of a ferrous solution.

The iron complexes are prepared by preparing partially hydrolyzed egg white protein, adding the iron source under acidic conditions, and then neutralizing.

The partially hydrolyzed egg white protein should be such that the molecular weight of the protein fractions is in the range of about 500 to about 10000; preferably about 2000 to about 6000. It is found that iron complexes which are prepared from intact egg white protein or extensively hydrolyzed egg white protein are not sufficiently strong. However, iron complexes prepared from partially hydrolyzed egg white protein are extremely stable.

The hydrolysis of the egg white protein may be carried out in one or more steps as is conventional. However, best results are obtained when the hydrolysis procedure includes an enzymatic hydrolysis step using an acid protease in an acidic medium. Suitable acid proteases are commercially available. Particularly suitable acid proteases may be obtained by the controlled fermentation of fungae such as *Aspergillus oryzae*. These proteases contain both endo-peptidases and exo-peptidases. An example of such an acid enzyme is VALIDASE FP-60 (obtainable from Valley Research, Inc., South Bend, Indiana).

The medium may be acidified by using a suitable food grade inorganic or an organic grade acid. Examples of acids which may be used are phosphoric, hydrochloric, sulfuric, lactic, malic, fumaric, gluconic, succinic, ascorbic, or citric. The most preferred acid is phosphoric acid. The pH may be selected to provide for optimum performance of the enzyme. The pH selected may be that at which the enzyme performs optimally. This information may be obtained from the supplier or by simple trial.

The hydrolyzed protein obtained after hydrolysis with the acid protease may be used in this form. However, the hydrolyzed protein may be further hydrolyzed if desired. For any further enzymatic hydrolysis steps which may be desired, any suitable enzyme may be used. Examples include but are not limited to ALCALASE, FLAVORZYME and NEUTRASE, (Novo Nordisk A/S, Novo Alle, Denmark), and PROZYME and PANCREATIN (Amano International Enzyme Co., Inc., Troy, VA). The enzymes may be acidic proteases, alkaline proteases or neutral proteases. Particularly suitable are alkaline proteases.

Prior to adding the iron source to the partially hydrolyzed egg white protein, the partially hydrolyzed egg white protein should be at an acidic pH of about 3.0 to about 5.5. If necessary, the pH may be adjusted by adding a suitable food grade inorganic or an organic grade acid as defined above. The most preferred acid is phosphoric acid.

The ferrous solution and the partially hydrolyzed egg white protein are then combined. This is preferably carried out under agitation with the ferrous

solution added to the partially hydrolyzed egg white protein; preferably slowly. The amount of the ferrous solution which is added may be selected to provide the desired ferrous loading. However, it is surprisingly found that the binding of the ferrous in the complex is related to the amount of ferrous bound. Optimum binding is obtained when the complex contains about 1% to about 2% or about 4.5% to about 10% by dried weight of iron. Of course, ferrous loads of more than 10% may be used but the binding, and hence the stability of the complex, may be slightly less.

After adding the iron source to the partially hydrolyzed egg white protein, the solution should be neutralized to promote the formation of a ferrous complex. However, the mixture should not be allowed to become basic to avoid precipitation and the formation of hydroxide ions. A pH in the range of about 6.5 to about 7.5 is recommended.

If necessary, an alkali may be added to neutralize the pH of the mixture. Any food grade alkali may be used for neutralization, including but not limited to sodium hydroxide, potassium hydroxide, ammonia hydroxide, magnesium hydroxide, sodium carbonate, sodium bicarbonate, potassium carbonate, and potassium bicarbonate. Ammonia hydroxide is preferred.

All steps are preferably carried out under agitation.

The complexes obtained may be used in liquid form as obtained. More preferably however, the complexes are dried to powder. The drying may be freeze drying or may be spray drying. Any suitable procedure for spray- or freeze-drying the complexes to powder may be used. Suitable procedures are known in the art.

In use, the complexes are included in the ingredients making up the desired foods or beverage and the ingredients processed in the normal way. Although the bioavailability of the iron may be slightly less than that of ferrous sulfate, it is found that it is well within acceptable limits. In most cases, the statistical difference in bioavailability is not significant. Further, it is found that the complexes are very stable and when used in foods and beverages, do not lead to increased discoloration or off-flavor generation. Moreover, it is found that the complexes do not increase processing problems such as fouling.

The complexes are particularly suitable for use in foods or beverages in liquid form; for example infant formula concentrates and ready-to-drink beverages such as chocolate and malted milk drinks. These foods or beverages usually undergo retorting or other sterilization as part of their processing and

hence the ability of the complexes to withstand harsh treatment provides a great improvement. However, the complexes may be used in other types of foods or beverages such as powdered beverages, infant formulas, and infant cereals.

The complexes may also be included in pet foods which usually contain lipids and vitamins.

Products which contain the complexes are perceived to have similar organoleptic properties and color as compared to unfortified products. This offers the advantage that products may be fortified without causing noticeable changes which may adversely affect consumer perception. Also, it is found that vitamin C is not degraded by the complexes. Hence the complexes may be used in products which are intended to be nutritionally balanced.

Specific examples of the invention are now described to further illustrate the invention.

Example 1

An amount of 1000 g of frozen egg white is added to a fermentor (Biostat® M) and allowed to thaw at room temperature. The pH is slowly adjusted to 3.0 using 85% H_3PO_4 under agitation. The solution is then heated to 42 °C. An amount of 2.5 g of an acid protease (VALIDASE FP60 obtained from Valley Research, Inc or South Bend, Indiana) is added and the solution allowed to react for 16 hours under low/medium agitation at a pH of 3.0 to 3.3. This acid protease is obtained from *Aspergillus oryzae* and contains both endo-peptidase and exo-peptidase.

After 16 hours of reaction, ammonium hydroxide (28%) is added to raise the pH to 7.4. An amount of 2.5 g of alkaline protease (ALCALASE 2.4L, obtained from Novo Nordisk A7S) is added and the temperature of the solution is raised to 50 °C under agitation. This protease is obtained from a strain of *Bacillus licheniformis* and contains mainly endo-proteinase. After 3 hours of reaction under low/medium agitation, the solution is cooled to room temperature. An amount of 43.5 g of 85% H_3PO_4 is added followed by an amount of 5.0 g of $FeSO_4.7H_2O$ in 50 ml of H_2O, both under agitation. The pH is then adjusted to 6.7 with 28% NH_4OH under agitation. The solution is then heated to a temperature of 90 °C for 10 minutes. The solution is then cooled to room temperature.

The liquid iron complex is collected.

Example 2

The process of example 1 is repeated. Then an amount of 90 g of maltodextrin M.D. 5 is added to the liquid iron complex under agitation. The mixture is then spray dried using an atomizing spinning disk spray-drier (T_{in} = 145 °C, T_{out} = 80 °C).

The powdered iron complex is collected.

Example 3

An amount of 1000 g of frozen egg white is added to a fermentor (Biostat® M) and allowed to thaw at room temperature. The pH is slowly adjusted to 3.0 using 85% H_3PO_4 under agitation. The solution is then heated to 42 °C. An amount of 2.5 g of an acid protease (VALIDASE FP60 obtained from Valley Research, Inc or South Bend, Indiana) is added and the solution allowed to react for 4 hours under low/medium agitation at a pH of 3.0 to 3.3.

After reaction, the solution is cooled to room temperature. An amount of 5.0 g of $FeSO_4.7H_2O$ in 50 ml of H_2O is added under agitation. The pH is then adjusted to 6.7 with 28% NH_4OH under agitation. The solution is then heated to a temperature of 60 °C for 10 minutes. The solution is then cooled to room temperature.

An amount of 90 g of maltodextrin M.D. 5 is added to the solution under agitation. The mixture is then spray dried using an atomizing spinning disk spray-drier (T_{in} = 145 °C, T_{out} = 80 °C).

The powdered iron complex is collected.

Example 4

The process of example 1 is repeated except that the egg white is subjected to hydrolysis for 6 hours. The powdered iron complex is collected.

Example 5

Four chocolate milk beverages are prepared by reconstituting a chocolate milk powder (QUIK, Nestlé USA, Inc) to a concentration of 8.5% by weight.

Each beverage contains 12.5 ppm of added iron in the form of a different iron complex of one of examples 1 to 4.

The beverages are placed into sealed 125 ml glass jars and autoclaved at about 121°C (250°F) for 5 minutes. The jars are cooled to room temperature and stored for 6 months.

The beverages are evaluated for physical stability, color and taste after 1, 2, 3, 4, 5 and 6 months. Taste is judged by a taste panel of 10 people. All beverages are judged to be without discoloration, sedimentation or coagulation and of a good flavor.

Example 6

Four chocolate milk beverages are prepared by reconstituting a chocolate milk powder (QUIK, Nestlé USA, Inc) to a concentration of 8.5% by weight. Each beverage contains 12.5 ppm of added iron in the form of a different iron complex of one of examples 1 to 4.

The beverages are pre-heated to about 80°C (175°F), heated to about 140°C (285°F) by steam injection, held at this temperature for 5 seconds, and cooled to about 80°C (175°F). The beverages are then homogenized at about 17/3.5 MPa (2500/500 psi), cooled to about 16°C (60°F) and filled in 250 ml Tetra Brik Aseptic® packages (Tetra Pak Inc., Chicago IL).

The beverages are evaluated for physical stability, color and taste after 1 day, 2 weeks, and 1 and 2 months. Taste is judged by a taste panel of 10 people. All beverages are judged to be without discoloration, sedimentation or coagulation and of a good flavor.

Example 7

Four chocolate milk beverages are prepared by reconstituting a chocolate milk powder (QUIK, Nestlé USA, Inc) to a concentration of 8.5% by weight. Each beverage contains 12.5 ppm of added iron in the form of a different iron complex of one of examples 1 to 4.

The beverages are pre-heated to about 80°C (175°F), heated to about 148°C (298°F) by steam injection, held at this temperature for 5 seconds, and cooled to about 80°C (175°F). The beverages are then homogenized at about

17/3.5 MPa (2500/500 psi), cooled to about 16°C (60°F) and filled in 250 ml Tetra Brik Aseptic® packages (Tetra Pak Inc., Chicago IL).

The beverages are evaluated for physical stability, color and taste after 1, 2, 3, 4, 5 and 6 months. Taste is judged by a taste panel of 10 people. All beverages are judged to be without discoloration, sedimentation or coagulation and of a good flavor.

Example 8

Six beverages are prepared; 3 by reconstituting a chocolate milk powder (QUIK, Nestlé USA, Inc) and 3 by reconstituting a malted powder (MILO, Nestlé Australia Ltd). Each beverage comprises 22.0 g of powder in 180 ml of boiling water. An iron complex of each of examples 2 to 4 is added to both a chocolate beverage and a malted beverage. The final iron concentrations in the chocolate beverages are 15.0 ppm and in the malted beverages are 25.0 ppm.

The beverage are stirred briefly and allowed to stand for 15 minutes at room temperature. After 15 minutes, beverages are judged by a taste panel of 10 people. No color change or off flavors are found when samples are compared to control samples without added iron.

Example 9

Three infant cereal meals are prepared by reconstituting 55 g of banana containing infant cereal (Nestlé USA, Inc) with 180 ml of boiling water. Iron complexes of examples 2 to 4 added to each cereal to provide 7.5 mg of iron per 100 g of cereal powder.

Each cereal meal is stirred briefly and allowed to stand for 15 minutes at room temperature. After 15 minutes, the cereal meals are judged by a taste panel of 10 people. No color change or off flavors are found when samples are compared to control samples without added iron.

Example 10

The bioavailabilities of the complexes are determined as follows:-

Animals:- The animals used are weanling male Sprague-Dawley rats aged 3 weeks (IFFA-CREDO, L'Arbresle, France).

Diets:- The control diet is an ICN Low-Iron diet (Soccochim SA, Lausanne, Switzerland) which has an iron content of 3 mg/kg. This diet is casein based and provides for the nutritional requirements of growing rats except for iron.

The experimental diets are:-

Diet A:- The control diet supplemented with $FeSO_4.7H_2O$ to provide 10 mg/kg iron.

Diet B:- The control diet supplemented with $FeSO_4.7H_2O$ to provide 20 mg/kg iron.

Diet 1:- The control diet supplemented with the complex of example 4 to provide 10 mg/kg iron.

Diet 2:- The control diet supplemented with the complex of example 4 to provide 20 mg/kg iron.

Diet 3:- The control diet supplemented with 10 mg/kg of the complex of example 2 to provide 10 mg/kg iron.

Diet 4:- The control diet supplemented with 20 mg/kg of the complex of example 2 to provide 20 mg/kg iron.

Analytical methods

1) Hemoglobin analysis is performed by anaesthetizing the rats with isoflurane and then drawing a sample of 200 µL of blood from the orbital venous plexus. Blood hemoglobin level in the sample is determined by the cyanmethemoglobin method (Hb kit MPR 3, Boehringer Mannheim GmbH, Germany), using an automated instrument (Hemocue, Baumann-Medical SA, Wetzikon, Switzerland). Commercial quality control blood samples (Dia-HT Kontrollblut, Dia MED, Cressier, Switzerland) having a range of hemoglobin levels are measured with all hemoglobin determinations.

2) Fe-bioavailability as compared to ferrous sulfate heptahydrate is evaluated using a slope-ratio calculation based upon hemoglobin levels. A multiple regression equation relates amounts of iron added to the hemoglobin

levels. The equation provides one straight line per diet which intercepts at zero dose. The bioavailability of the iron source relative to ferrous sulfate heptahydrate is then calculated as the ratio of the two slopes. The ratio is multiplied by 100 to provide the relative bioavailability value.

Procedure:- Rats are housed individually in polycarbonate cages, fitted with stainless steel grids. The animals are allowed free access to distilled water. To render the rats anemic, the rats have *ad libitum* access to the control diet for 24 days. Fresh diet is supplied daily. Spoiling of diet by rats is reduced by covering the diet with a grid.

After 24 days, hemoglobin and weight is determined. Seventy rats with hemoglobin levels between 4.5 and 5.8 mg/dl are randomized into 7 groups of 10 having approximately equal mean hemoglobin and body weight. Each group of animals is fed one of the experimental diets for 14 days. The rats are fed the diets *ad libitum* beginning with 20 g/day at day 0. The rats have free access to distilled water. Individual food consumption is measured daily. After 14 days, the rats are weighed and hemoglobin is determined.

Results

Mean food consumption and iron intake is not affected by the type of iron source. However the rats receiving no added iron ate less than those receiving iron. The rats consuming diets with 20 mg/kg of added iron consume slightly more than those receiving diets with 10 mg/kg iron.

Weight increase of the rats is not affected by the type of iron source. However, the rats receiving no added iron gained less weight than those receiving iron. The rats receiving diets with 20 mg/kg iron gain slightly more weight than those receiving the diets with 10 mg/kg iron.

The blood hemoglobin levels at the start and at the end of the period are shown in the table below.

Mean hemoglobin values; (Standard Deviation)

Diet	Added Fe (mg/kg)	Initial hemoglobin (g/dl)	Final hemoglobin (g/dl)	Difference (g/dl)
Control	0	5.12 (0.42)	4.88 (0.43)	-0.24 (0.20)
A	10	5.12 (0.41)	8.66 (0.81)	3.54 (0.65)
B	20	5.12 (0.40)	11.53 (0.86)	6.41 (0.82)
1	10	5.12 (0.40)	7.90 (0.54)	2.78 (0.41)
2	20	5.13 (0.39)	11.15 (0.57)	5.92 (0.54)
3	10	5.13 (0.37)	8.36 (0.47)	3.23 (0.34)
4	20	5.12 (0.38)	11.51 (0.79)	6.39 (0.65)

The relative bioavailabilities are as follows:-

Diet	Relative Bioavailability
1, 2	90
3, 4	98
A, B	100

The bioavailabilities of all of the Fe-protein complexes are similar to that of ferrous sulfate. A relative bioavailability value of less than 91% is taken to be significantly less than the reference. Therefore, from a statistical point of view, the relative bioavailability values of the iron complexes of example 2 are similar to that of ferrous sulfate. However, from a practical viewpoint, all of the complexes have very good bioavailability.

We Claim

1. An iron-protein hydrolysate complex which comprises ferrous ions chelated to partially hydrolyzed egg white protein having a molecular weight in the range of about 500 to about 10'000.

2. A complex according to claim 1 in which the partially hydrolyzed egg white protein has a molecular weight in the range of about 2'000 to about 6'000.

3. A complex according to claim 1 in which the partially hydrolyzed egg white protein is microbial protease hydrolysate.

4. A complex according to claim 3 in which the microbial protease is obtained from *Aspergillus oryzae* and contains both endo-peptidase and exo-peptidase.

5. A complex according to claim 1 in which partially hydrolyzed egg white protein is a microbial protease hydrolysate obtained by hydrolyzing egg white protein with a protease obtained from *Aspergillus oryzae* and containing both endo-peptidase and exo-peptidase, and a protease obtained from *Bacillus licheniformis* and containing endo-proteinase.

6. A complex according to claim 1 which contains about 1% to about 2% or about 4.5% to about 10% by dried weight of ferrous ions.

7. A complex according to claim 1 which is stable at neutral pH but disassociates at a pH below about 3.

8. An iron-protein hydrolysate complex which comprises ferrous ions chelated to partially hydrolyzed egg white protein which is a microbial protease hydrolyzate; the microbial protease both endo-peptidase and exo-peptidase.

9. A complex according to claim 8 in which the partially hydrolyzed egg white protein has a molecular weight in the range of about 500 to about 10'000.

10. A complex according to claim 9 in which the partially hydrolyzed egg white protein has a molecular weight in the range of about 2'000 to about 6'000.

11. A complex according to claim 8 which contains about 1% to about 2% or about 4.5% to about 10% by dried weight of ferrous ions.

12. A complex according to claim 8 which is stable at neutral pH but disassociates at a pH below about 3.

13. An iron-protein hydrolysate complex which comprises ferrous ions chelated to partially hydrolyzed egg white protein; the complex containing about 1% to about 2% or about 4.5% to about 10% by dried weight of ferrous ions.

14. A complex according to claim 13 in which the partially hydrolyzed egg white protein has a molecular weight in the range of about 500 to about 10'000.

15. A complex according to claim 13 in which the partially hydrolyzed egg white protein has a molecular weight in the range of about 2'000 to about 6'000.

16. A complex according to claim 13 in which the partially hydrolyzed egg white protein is microbial protease hydrolysate.

17. A complex according to claim 13 in which the fungal protease contains both endo-peptidase and exo-peptidase.

18. A complex according to claim 13 which is stable at neutral pH but disassociates at a pH below about 3.

19. A sterilized liquid beverage which contains lipid and a stable iron fortification system, the iron fortification system comprising an iron-protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein.

20. A beverage according to claim 19 which is a chocolate containing beverage.

21. A sterilized liquid beverage which contains polyphenols and a stable iron fortification system, the iron fortification system comprising an iron-protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein.

22. A beverage according to claim 21 which is a tea beverage.

23. A beverage powder which contains lipid and a stable iron fortification system, the iron fortification system comprising an iron-protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein.

24. A beverage powder according to claim 23 which contains cocoa.

25. A process for preparing an iron fortification system, the process comprising:

enzymatically hydrolyzing an egg white protein using a microbial protease to provide a partially hydrolyzed egg white protein;

adding a ferrous source to the partially hydrolyzed egg white protein under acidic conditions; and

raising the pH to 6.5 to 7.5 for forming a ferrous-hydrolyzed egg white protein complex as the iron fortification system.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/01743

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23L1/304 A23G1/00 A23J3/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23L G03C B01D C07F C01B C05D A23G A23J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
15 June 2000	28/06/2000

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Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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